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(57) Abstract

The invention relates to improvements of energy-transfer based homogeneous assays, which use time-resolved fluorometry in detection. The specific improvements relate to the type of lanthanide chelate labels used as energy donors, optimized energy acceptors for defined assays, the way energy transfer is measured using optimized filters and time windows, ways to correct all possible interferences derived from samples, use the assay for multi-component analysis and development of simplified assay protocols.

HOMOGENOUS LUMINESCENCE ENERGY TRANSFER ASSAYS

FIELD OF THE INVENTION

The present invention relates to improvements of energy-transfer based homogeneous assays, which use time-resolved fluorometry in detection. The specific improvements relate to the type of lanthanide chelate labels used as energy donors, optimized energy acceptors for defined assays, the way energy transfer is measured using optimized filters and time windows, ways to correct all possible interferences derived from samples, use the assay for multi-component analysis and development of simplified assay protocols.

BACKGROUND OF THE INVENTION

The publications and other materials used herein to illuminate the background of the invention, and in particular, cases to provide additional details respecting the practice, are incorporated by reference.

A great variety of assays based on bioaffinity or enzymatically catalysed reactions have been developed to analyze biologically important compounds from various biological samples (such as serum, blood, plasma, saliva, urine, feces, seminal plasma, sweat, liquor, amniotic fluid, tissue homogenate, ascites, etc.), samples in environmental studies (waste water, soil samples) industrial processes (process solutions, products) and compound libraries (screening libraries which may comprise organic compounds, inorganic compounds, natural products, extracts of biological sources, biological proteins, peptides, or nucleotides, and so on). Some of these assays rely on specific bioaffinity recognition reactions, where generally natural biological binding components are used to form the specific binding assay (with biological binding components such as antibodies, natural hormone binding

proteins, lectins, enzymes, receptors, DNA, RNA or PNA) or artificially produced binding compounds like genetically or chemically engineered antibodies, molded plastic imprint (molecular imprinting) and so on. Such assays generally rely on a label to quantitate the formed complexes after recognition and binding reaction and suitable separation (separations like precipitation and centrifugation, filtration, affinity collection to e.g. plastic surfaces such as coated assay tubes, slides of microbeads, solvent extraction, gel filtration or other chromatographic systems, and so on). The quantitation of the label in free or bound fraction enables the calculation of the analyte in the sample directly or indirectly, generally through use of a set of standards to which unknown samples are compared.

The separation and washing needed in most of these assays make them labor intensive, slow and difficult to automate. Furthermore the end point measurement does not allow gathering of kinetic information (associations/ dissociation rates). In cases of low affinity bindings, the affinity may be so low that no physical separation can be applied without destroying the binding (low affinity receptors). Particularly in areas, like screenings (high throughput screening) there is a constant demand for simpler assays, simplified protocols, which would make automation easier and increase the throughput. That can be accomplished with homogeneous or non-separations assays. Homogeneous biomedical assays are defined as assays taking place in one homogeneous phase. It means that no separate phases (such as solid phase catching reagents), and no separation is used prior to measurement. That requires a signal production system that responds to the binding assay in a way making its direct monitoring possible. Systems know prior art are e.g. fluorescence polarization assays applied for small molecular compounds, enzyme-monitored immunoassays (marketed by Syva), various fluorescence quenching or enhancing assays (for an review see e.g. Hemmilä, Applications of Fluorescence in Immunoassays,

Wiley, NY, 1991). Another category of simplified assay technologies are non-separation assays, which, similarly to homogenous assays, avoid separation and washing steps. A good example of such a technology is the scintillation proximity principle marketed by Amersham, which is based on short distance penetration of radiation particles in assay medium and solid scintillator coated with catching reagents (Udenfriend et al, (1985) Proc Natl Acad Sci, 82, 8672 and Anal Biochem, (1987) 161, 494).

Regardless of a great number of homogeneous assay designs published to day, there are no assays, where the versatility and sensitivity would match those of a good separation assay. The reason to that is manyfold relating to e.g. the different way homogeneous, versus heterogeneous, assay has to be optimized, the control of low affinity non-specific bindings, and the limitations of applicability of most of the existing homogenous assay techniques. In addition, the conventional homogeneous fluorometric assays are very vulnerable to background interferences derived from various components in the samples. Fluorescence polarizations assays are interfered by low affinity nonspecific bindings (e.g. probe binding to albumin) and autofluorescence of samples.

Time-resolved fluorometry (time resolution in time-domain at micro- or millisecond range) is a perfect measuring regime for homogeneous assays, because it can totally discriminate the background fluorescence derived from organic compounds when long enough delay times (time between pulsed excitation and starting of emission recording) are used (for a review see. e.g. Hemmilä (1991); Gudgin Dickinson et al, (1995) J Photochem Photobiol 27, 3). In addition to separation based assays, also a number of homogeneous time resolved fluorometric assays have been described and patented (Mathis (1995) Clin Chem, 41, 1391; Selvin et al. (1994) Proc Natl Acad Sci, USA, 91, 10024)

with their limitation and drawbacks. The aim of the present invention is i.a. to provide an improvement in time-resolved homogeneous assay principle based on specific energy transfer between a long life-time chelate-based donor probe and short life-time (or non-emitting) acceptor molecule.

Resonance Energy Transfer

The Förster type of nonradiative dipole-dipole energy transfer (Förster (1948) Ann Physik, 6, 55) takes place between two molecules in condition where their energies (emission of donor with absorption of acceptor) overlap and they are at a distance less than 20 nm. The energy transfer requires a proper orientation of the oscillations of the molecules. The energy transfer efficiency (Φ_{ET}) is given in the equation:

$$\Phi_{ET} = 1/[1 + (r/R_0)^6]$$

where r is the distance between the donor and acceptor molecules and R_0 is a distance parameter characteristic of the donor-acceptor pair and the medium between them.

Fluorescence resonance energy transfer (FRET) has been applied e.g. as an spectroscopic ruler in structural studies to measure distances within a macromolecule (Stryer and Haugland (1967) Proc Natl Acad Sci, USA, 58; 719). In addition to resonance energy transfer, there are also other energy transfer reactions, like simple radiative (where acceptor absorbs the light emitted by donor), collisional energy transfer, exchange (Dexter (1953) J Chem Physics, 21, 836) and exciton migration (in crystals). In addition, the donor emission can be quenched by numerous ways with a number of unrelated compounds having an deactivating effect on some of the donor's energy levels.

Ullman was the first to describe application of Förster-type non-radiative energy transfer in bioanalytical assays based on antibody recognition reaction (Ullman, Scharzberg and Rubenstein (1967) J Biol Chem, 251; 4172) marketed as FETIA (fluorescence energy transfer immunoassay) by Syva Co. (US 3,996,345). Development of suitable energy donor-acceptor pairs are well described in their various reviews (Ullman and Khanna (1981) Methods Enzymol, 74; 28; Khanna and Ullman (1980) Anal Biochem, 108, 156). FETIA primarily applies xanthene dyes and derivatives of fluorescein as donor and rhodamines as acceptors. A great number of alternative probe pairs have since been developed and applied in immunoassays (for a review see Hemmilä 1991, chapter 8.3.4) including practically non-fluorescent derivatives of fluorescein, long life-time delayed fluorescence emittive eosin (Thakrar and Miller (1982) Anal Proc, 19, 329), long lifetime fluorescent pyrene (Morrison (1988) Anal Biochem, 174, 101) and non-emittive charcoal. FRET has since got wide applications in basic research and in DNA hybridizations (see e.g. Morrison et al (1989) Anal Biochem, 183, 231; Parkhurst et al (1995) Biochemistry, 34, 285) and other assays were association, dissociation or distances are to be measured.

The use of temporal discrimination (time-resolution) to avoid the effect of direct excitation of acceptor molecule was described by Morrison (Morrison (1988) Anal Biochem, 174, 101) with organic donor-acceptor pairs of different decay times using pyrene as the long excited state donor, pulsed laser for excitation and phycoerythrin as the short decay-time acceptor (US 4,822,733). In addition to immunoassays, the time-resolved energy transfer principle is applied for homogeneous solution hybridization using fluorescein (Morrison et al (1989) IIIrd International Symposium on Quantitative Luminescence Spectrometry in Biomedical Sciences, Ghent Belgium) as acceptor. The long excited state provides the advantage, that specific energy

transfer can be followed using a delay time, during which the emission of directly excited acceptor is decayed off. The combination of different decay times exploited in time-resolved fluorometry will provide a clear advantage over FRET technologies employing conventional short decay probes.

Luminescent (fluorescent) lanthanides have long been used as tools for DNA and protein structural studies (See e.g. Brittain (1985) Bioinorganic Luminescence Spectroscopy, Schulman (ed), Molecular Luminescence Spectroscopy, Wiley, NY; Bünzli and Choppin (1989) Lanthanide Probes in Life, Chemical and Earth Sciences, Theory and Practice, Elsevier Science Publisher, Amsterdam), applied e.g. for RNA, ribosomes, DNA and chromatin, termolysin and transferrin, calmodulin, ATPase, nerve membranes, erythrocyte membrane proteins, phospholipids and liposomes and their fusions (for an review see Hemmilä 1991). The energy transfer between lanthanides attached to different sites of a calcium binding protein, or DNA, have been used as spectroscopic ruler to measure the locations of ion-binding sites, e.g. with calmodulin using energy transfer between Eu and Nd (Horrocks and Tingey (1988) Biochemistry, 27, 413). Similarly FRET has been used in chelate studies by measuring energy transfer from Tb to Eu (Brittain (1978) Inorg Chem, 17, 2762).

Lanthanides and their chelates are good candidates for time-resolved FRET experiments. Firstly, they have exceptionally long excited state lifetime (Weissman (1942) Chem Phys, 10, 214; Whan and Crosby (1962) Mol Spectrosc, 8, 315). The energy transfer takes place only between transitions that are electric dipole. The major transition in the highly fluorescent Eu chelates, (transition $^5D_0$ - $^7F_2$) at 612-620 nm is electric dipole "forced" (Bünzli (1989) Lanthanide Probes in Life, Chemical and Earth Sciences. Theory and Practice, Bünzli and Choppin (ed) Elsevier

Science, Publisher, Amsterdam) and can donate energy. The transition $^5D_0$ - $^7F_1$ producing emission at 590-595 nm is, however, magnetic dipole and can not transfer energy (Dexter, J Chem Phys 21; 836, 1953). In addition, lanthanide emission has isotropic dipolic moment, and hence the orientation factor becomes less ambiguous (Ando et al (1992) Biochim Biophys Acta, 1102, 186).

Fluorescent lanthanide chelates have been used as energy donors already since 1978 by Stryer, Thomas and Meares. For example a Tb dipicolinate chelate reported to have the critical distance (R_o) of 6.57 nm for rhodamine, 4.46 nm for eosin and 4.46 nm for NBD (Thomas et al (1978) Proc Natl Acad Sci, 75; 5746). Typically in an efficient energy transfer, the overall decay shortens, as shown in the above system, from 2.22 ms to 0.12 ms. Meares et al (1981, 1992) measured enzyme bound rifamycin using Tb-phenyl-EDTA as energy donor (Biochemistry 20; 610; Biochemistry 22; 6247). In addition to fluorescent compounds, also nonluminescent acceptors have been reported, such as Co (III) and Co (II) (Cronce and Horrocks (1992) Biochemistry 31, 7963). Eu and Tb chelates have also been tested as energy acceptors, Eu for coumarin derivative and Tb for salicylate derivative (Clark et al (1993) Anal Biochem 210, 1). The great number of available non-luminescent energy acceptors (quenchers) is an additional advantage for lanthanide chelates. They can be quenched by metallic ions, nitrite (Tanaka et al (1993) J Photochem Photobiol A: Chem, 74, 15), other paramagnetic metallic ions and their chelates, free radicals (Matko et al (1992) Biochemistry 31, 703) and so on.

The use of lanthanides as fluorescent labels in various forms of bioaffinity assays are well documented in a great number of reviews and patents (see for example Hemmilä 1991, Gudgin Dickson 1995). Examples of the early ideas about a 'workable' chelate label can be found e.g. in

following patents (US 4,374,120; 4,432,907; 4,965,211; 4,341,957; 4,058,732 and 4,283,382). A heterogeneous assay system based on non-luminescent labelling chelates (US 4,808,541) and a dissociative fluorescence enhancement (US 4,565,790) has got the widest applications. That system, as well as system based on fluorogenic ligand and after-assay cationic saturation (EP-A 354847 and US 4,772,563), does not suit for homogeneous assays. There are, however, a great number of stable fluorescent chelates, described in patents and articles, which could be used in time-resolved FRET assays, such as mentioned in following US patents: 4,761,481; 5,032,677; 5,055,578; 5,106,957; 5,116,989; 4,761,481 4,801,722; 4,794,191; 4,637,988; 4,670,572; 4,837,169 and 4,859,777. The preferred chelate is composed of nona-dentate chelating ligand, such as terpyridine (EP-A 403593; US 5,324,825; US 5,202,423, US 5,316,909), terpyridine analogue with one or two five-membered rings (e.g. pyrazole, thiazole, triazine) (US Appl 08/548,174 and PCT/FI91/00373). Very well suited chelates are also mentioned in following articles (Takalo et al (1994) Bioconjugate Chem, 5, 278; Mukkala et al (1993) Helv Chim Acta, 76, 1361; Remuinnan et al (1993) J Chem Soc Perkin Trans, 2, 1099; Mukkala et al (1996) Helv Chim Acta, 79, 295; Takalo et al (1996) Helv Chim Acta, 79). Examples of other long decay time labels suitable for the present invention are the phosphorescent complexes of porphyrin derivatives with Pd and Pt (WO 94/10568).

Use of long decay-time lanthanide chelates in various types of homogeneous binding assays are also described, based e.g. on mixed-ligand chelate (US 4,587,223), environmentally sensitive chelate (EP 324323) and a chelate as enzyme substrate (EP 374221).

Since the development of the fluorescence resonance energy transfer based homogeneous assays in 1976, they have

obtained wide applications in diagnostics, commercialized by Syva corporation (US 3,996,345). Use of lanthanide chelates as specific labels in time-resolving fluorometric systems was first described in 1970's by Leif (Leif et al (1976) Automation of Uterine Cancer Cytology 1, GL Weid, GF Bahar and PH Bartels (ed) Tutorial of Cytology, Chicago IL) and Wieder (Wieder (1978) Proc. 6th Int. Conf. On Immunofluorescence and related staining techniques, W Knapp, K Holubar and G Wick (eds), North-Holland, Amsterdam; US 4,058,732, DE 2,628,158) followed by the development of working diagnostic systems in 1980's (US 4,565,790). On the other hand, fluorescent lanthanides were used for fluorescence resonance energy transfer experiments already in 1978 by Stryer et al (Thomas et al (1978) Proc Natl Acad Sci, 75, 5746; Stryer et al, Annual Review of Biophysics and Bioengineering; Mullins LJ (ed) Annual Reviews, Inc; Palo Alto, CA, 1982, vol 11,p 203). Accordingly, their use as long excited state probes for FRET immunoassays is obvious, but still an object of patent applications. Wieder and Hale have obtained a patent on homogeneous time-resolved immunoassays based on various techniques, including energy transfer and quenchings (EP-A 272320 and WO 87/07955) and Stavrianopoulos (EP-A 242527, 1987) in an assay based on the use of C1q and FRET. Hoffman La Roche has patented a specific application using lumazine-type of chromophore as donor and a ruthenium chelate as acceptor for interactions between nucleic acids (EP 439036) and Cis Bio International applied for an assays based on Eu or Tb cryptates as donors for phycobiliproteins (WO 92/01225).

The principle of a time-resolved homogeneous assay based on a specific energy transfer between a long-life time donor and a short life-time emitting acceptor molecule can be summarized as follows: The emission duration of a short excited state acceptor, excited during the excited life-time of a long-decay time donor through the mechanism of energy transfer, is longer than the decay time of the

directly excited acceptor. This fact permits the use of temporal discrimination (time-resolution) to avoid the luminescence from directly excited acceptor. In a homogenous assay based on energy transfer the long-lived donor is excited with a light pulse (A) (Figure 1). The luminescence emission of the energy-transfer excited acceptor is measured (D) after a suitable delay (C), to avoid interference derived from directly excited acceptor (B). The long-lived emission from the donor (DE) is avoided by measuring the emission of the energy-transfer excited acceptor (AE) at a wavelength where the donor emission is absent or negligible (Figure 2). Hence, in homogenous bioaffinity assay (receptor-ligand binding, hybridisation reaction, immunobinding, enzyme substrate binding etc.) the association or dissociation of donor-acceptor pairs can be followed by measuring the increase or decrease, respectively, in the signal from the energy-transfer excited acceptor.

OBJECTS AND SUMMARY OF THE INVENTION

An object of the present invention is to provide a luminescence energy transfer assay comprising a first group labelled with an energy donor and a second group labelled with an energy acceptor, wherein the acceptor is either a short excited state lifetime luminescent label or a non-luminescent label. The increase or decrease, respectively, in energy transfer from the donor label to the acceptor label resulting from shortening or lengthening, respectively, of the distance between said labels, is measured. According to the invention, the donor is a luminescent lanthanide chelate.

According to one aspect of the invention the assay is based on a reaction resulting in lengthening of the distance between the labelled donor and acceptor groups. According to this invention, the donor luminescent lanthanide chelate has a long excited state lifetime.

According to another aspect of the invention the assay is based on a reaction resulting in either shortening or lengthening of the distance between the labelled donor and acceptor groups and the donor is a luminescent lanthanide chelate having a long excited state lifetime. According to this aspect of the invention the long excited state lifetime luminescent lanthanide chelate has one or more of the following properties:

- a high luminescence yield,
- an excited state lifetime of 1 ms or more, and
- an emission distribution optimized for energy transfer.

According to yet another aspect of the invention, two or more analytes are simultaneously measured, wherein each analyte is measured by a specific donor-acceptor pair.

According to yet another aspect of the invention, the luminescent or non-luminescent label of the acceptor is attached to a solid carrier providing a large surface to which said second group is bound or can be bound via suitable covalent or non-covalent means.

According to yet another aspect of the invention, the acceptor is lipophilic compound anchored to a cell membrane or cell membrane fragment or the acceptor is made lipophilic by conjugating with a long chain hydrocarbon such as a fatty acid.

According to yet another aspect of the invention, the instrument used to measure energy transfer is able to follow multiple parameters simultaneously or consecutely, such as absorbances at the wavelenghts of excitation or emissions, luminescence of different durations, scattering and luminescence decay times, in order to monitor and correct any interferences the sample matrix may have to excitation, energy transfer or emissions.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the luminescence versus time after donor excitation with a light pulse (A) of directly excited acceptor (B) and the luminescence emission of the energy-transfer excited acceptor (D) after a suitable delay (C).

Figure 2 shows the luminescence intensity versus emission wavelength of donor (DE) and acceptor (AE).

Figure 3 shows a standard curve for the homogenous energy transfer assay of ßhCG (ß-subunit of human chorionic gonadotropin).

Figure 4 shows a standard curve for the homogenous energy transfer assay of PSA (prostate specific antigen).

Figure 5 shows a standard curve for the homogenous energy transfer assay of ßhCG using indirect labelling.

Figure 6 shows a standard curve for the homogenous energy transfer assay of ßhCG using indirect labelling and an acceptor different from that of Figure 5.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention relates to improvements of energy-transfer based homogeneous assays, which use time-resolved fluorometry in detection. The specific improvements relate to the type of lanthanide chelate labels used as energy donors, optimized energy acceptors for defined assays, the way energy transfer is measured using optimized filters and time windows, ways to correct all possible interferences derived from samples, use the assay for multi-component analysis and development of simplified assay protocols.

The terms "first group" and "second group" shall be understood to include any component such as bioaffinity

recognition component (in reactions where the distance between the groups decreases, e.g. in bioaffinity reactions) or a part of a molecule or substrate (e.g. the distal ends of a peptide molecule the cleavage of which will separate the two labelled groups from each other).

The term "luminescence" shall cover fluorescense, phosphorescence, chemiluminescence, bioluminescence and electro-generated luminescence, photoluminescence, radioluminescence, sonoluminescence, thermoluminescence and triboluminescence.

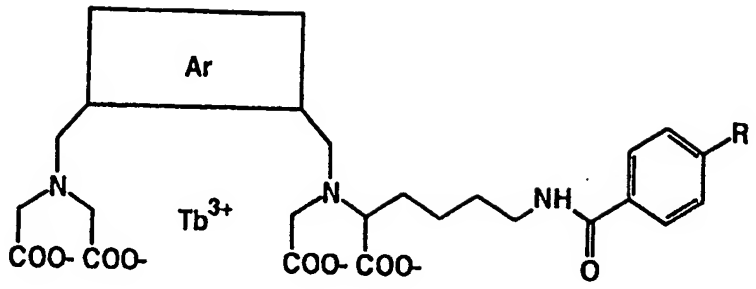
A preferred arrangement in assays, where association is to be measured is to use luminescent, short decay time acceptor and long decay time lanthanide chelate based donor and follow the emission of acceptor molecule using a delay time in the time-resolved fluorometry to avoid the interference of acceptors direct luminescence (emanating from direct excitation of acceptor). It is desirable to construct the assay in a way that acceptor molecules are in excess (with time-resolved mode, their interference is negligible) and the association of binding reagents creates an increase in signal.

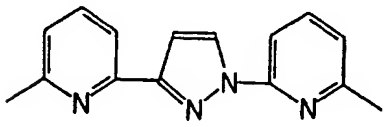
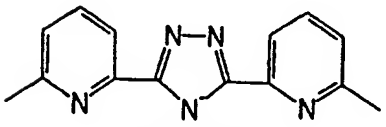
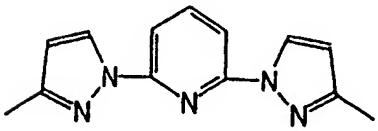
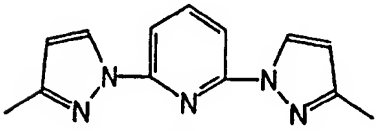
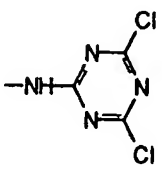
For such a system the preferred chelate label has to have high luminescence yield ($\epsilon x \Phi$ >2000), long excited state lifetime (preferably over 1 ms) and emission distribution optimized for energy transfer. The ligand field around the chelated ion has to be such that e.g. with Eu chelates over 70 % of emission is at $^5D_0$ - $^7F_2$ (at 610 - 620 nm range) and not at 590 nm range (compare e.g. emissions of Eu cryptate, WO 92/01225 and those of bis-iminoacetate derivatives of terpyridines, US 5,324,825; US 5,202,423 and US 5,316,909). In preferred chelates the useless magnetic dipole transition at 590 nm and emission around 700 nm are suppressed (Li and Selvin, J Amer Chem Soc 117; 8132, 1995). Particularly good chelates for the present application are Eu chelates formed with multichromogenic

polycarboxylates, having high molar absorption coefficient (ϵ), very long excited state lifetime and good quantum yield (Φ) (Takalo et al. Helv Chim Acta 79; 789, 1996). In addition to Eu, Tb is particularly promising energy donor, when its highly luminescent chelates are used. A preferred Tb chelate is composed of terpyridine derivatives containing the binding side at the iminodiacetate group (Mukkala et al, J Alloys Compounds 225; 507, 1995) or otherwise a binding arm well isolated from the light absorbing aromatic structure. Particularly good chelates for that applications are terpyridine derivatives where one or two pyridine rings are replaced with pyrazole (US 08/548,174) or triazole and thiazole rings (PCT/FI91/00373). In addition to Eu and Tb, the use of Sm would give the possibility to make double- or triple-label homogeneous energy transfer assays. Sm has the advantage, that it can donate energy at a rather high wavelength, the major emission of a highly luminescent chelate being at 643 nm, giving the opportunity to continue with the wavelength scale up to near IR (a good collection of near-IR emitting fluors have become commercially available from different sources). A preferred stable chelate of Sm is composed of multiple forms of 1,3-diketones, such as described by Savitsky (Savitsky et al, SPIE 2388; 429, 1995). An alternative third choice (third label) is the phosphorescent Pt or Pd coproporphyrins emitting a long lifetime phosphorescence at 650-660 nm (WO 94/10568).

A particularly preferred group of long excited state lifetime lanthanide chelates are Compounds 1 to 12 the structure of which is dislosed in Scheme 1 to 5. In the schemes λ_{exc} means excitation wavelength, τ decay time and $\epsilon \times \Phi$ luminescence yield. The values given in the schemes are measured from the compound coupled to a protein.

SCHEME 1



Ar	R	λ_{exc} [nm]	τ [µs]	$\varepsilon \cdot \Phi$
 COMPOUND 1	—N=C=S	328	1350	3860
 COMPOUND 2	—N=C=S	318	2720	4560
 COMPOUND 3	—N=C=S	315	2930	3770
 COMPOUND 4		310	2660	4050

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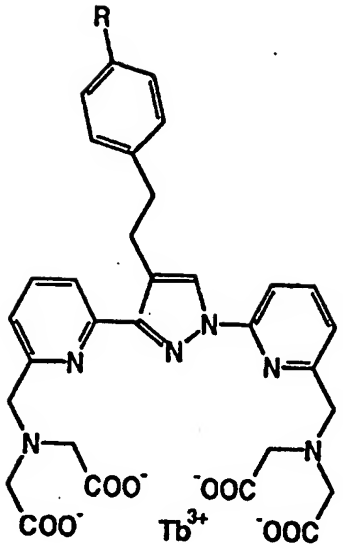
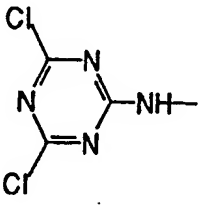


R	λ_{exc} [nm]	τ [μs]	ε · Φ
	333	1260	2060
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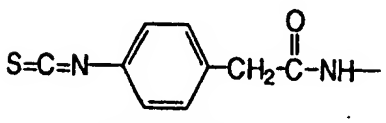
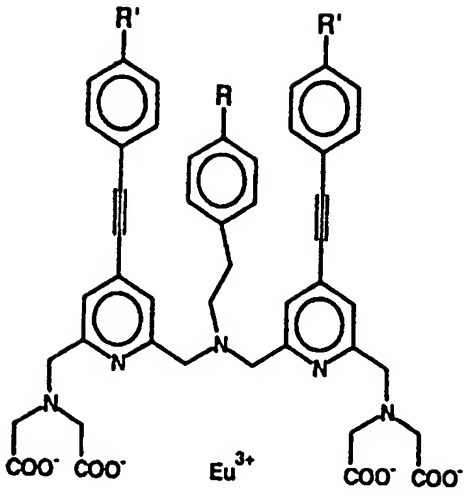
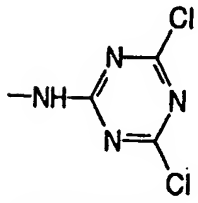
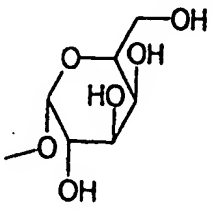
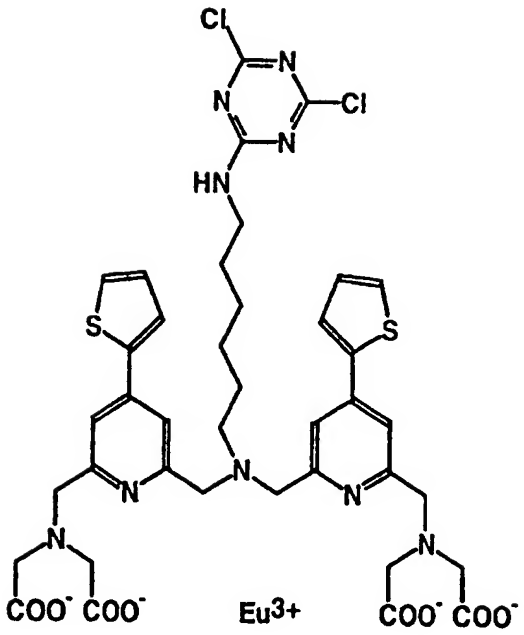
	336	1110	3690
COMPOUND 6			

SCHEME 3



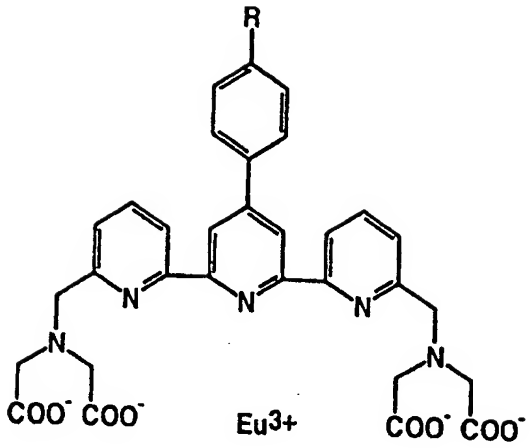
R	R'	λ_{exc} [nm]	τ [μs]	$\varepsilon \cdot \Phi$
—N=C=S	—OCH_2COO^-	335	1060	5950
COMPOUND 7				
	—OCH_2COO^-	336	1040	5190
COMPOUND 8				
—N=C=S		330	1000	3790
COMPOUND 9				

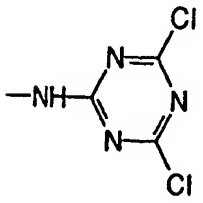
SCHEME 4



	λ_{exc} [nm]	τ [µs]	$\varepsilon \cdot \Phi$
COMPOUND 10	319	1050	4370

SCHEME 5



R	λ_{exc} [nm]	τ [μs]	$\varepsilon \cdot \Phi$
—N=C=S	340	1400	2100
COMPOUND 11			
	340	1590	2600
COMPOUND 12			

A preferred acceptor molecule for association assay is highly luminescent (with quantum yield as near unity (1) as possible) with high molar absorption coefficient (preferably over 100 000). The absorption of the acceptor has to overlap with the energy-donating emission of donor, i.e. at 611-620 nm for Eu, 545 nm for Tb, 643 nm for Sm. A preferred feature of the acceptor is its sharp emission at a wavelength where donor does not have any emission (such as 574-575 nm for Tb, 660-670 nm for Eu and around 675 for Sm). The decay-time of the acceptor should be below 1µs. In addition the acceptor has to be attached to binding partners, either directly or indirectly (e.g. via anti-binder antibody, lectin, avidin etc.), covalently or noncovalently. As covalently conjugated probes, e.g. those of xanthene dyes (rhodamine, tetramethylrhodamine, Texas Red™ (see e.g. Hemmilä, 1991, Haugland, Handbook of Fluorescent Probes and Research Chemicals, Molecular Probes 1992-1994, Mayer and Neuenhofer, Angew Chem Int Ed Engl 33; 1044, 1994), some carbocyanines (Cy3.18) (Mujumbar et al, Bioconjugate Chem 4; 105, 1993, Southwick et al, Cytometry 11; 418,1990) and even some porphyrins (Camman, DE 4,216,696) can be used. B-phycoerythrin, due to its very high molar absorption coefficient (2,410,00 at 546 nm) and quantum yield (0.98) makes it very suitable for energy transfer assays, despite its large size (MW 240,000).

A good acceptor for Eu should have absorption maximum at 611-620 nm range. Good acceptor for that wavelength range can be found particularly from the group of IR emittive dyes (for reviews see Haugland Molecular Probes, Miller, Spectroscopy Europe 5; 34, 1993, Patonay and Antoine, Anal Chem 63; 332A, 1991, Southwick et al, Cytometry 11; 418, 1990, Mujumbar et al, Cytometry 10; 11, 1989 and Mujumbar et al, Bioconjugate Chem 4; 105, 1993, Papkovsky, Appl Fluorescence Technol III; 16, 1991, Schindele and Renzoni, J Clin Immunoassay 13; 182, 1993). These types of compounds include phthalocyanines, porphyrins, cyanine dyes (Cy-5,

Cy5,18, Cy5,29) (Selvin et al, J Amer Chen Soc 116; 6029, 1994, Mujumbar et al, Bioconjugate Chem 4; 105, 1993), conjugated xanthenes (rhodamine 800) (Imasaka et al, Anal Chem 61; 2285, 1989), squarates (Chang, EP176,252), methylene blue, Nile blue and oxazines (Imasaka et al, Anal Chem 61; 2285, 1989), indocyanine green (Imasaka et al, Anal Chem 62; 363A, 1990). Some of the phycobiliproteins are good candidates also for Eu acceptors, particularly A-PC, C-PC and R-PC. A preferred energy acceptor for Sm or for coproporphyrins are the IR emittive dyes (see reviews mentioned above).

A preferred way to ascertain small distances between donor probe labelled ligand or binding reagent and acceptor probe labelled binding reagent is to use activated probes coupled directly to binding reagent (e.g. acceptor labelled receptor protein, antibody or other binding protein). An alternative way is to use indirect labelling, using e.g. anti-binder (such as anti-receptor) antibodies labelled with the acceptor, use of biotinylated binder and acceptor labelled (strept)avidin or to employ other bioaffinity reactions to bring acceptor molecules in the vicinity of actual binding site, where the donor-labelled component either directly or indirectly will be bound.

A preferred way with impure, membrane-bound receptors is to use lipophilic membrane probes and stain all the membranes near or around the receptor with acceptor molecules. Suitable probes attached to long aliphatic hydrocarbon chain, such as e.g. oxacarbocyanine (Molecular Probes) are suitable for such staining.

A further alternative, which would avoid separate labelling of binding components for each particular assay, is to use solid carriers (polymers, ceramics or glass or the like) such as universal catching surfaces containing high concentration of acceptor molecules. Suitable solid carriers can be e.g. beads or particles with a diameter up

to 1500 µm or any solid surface. Microbeads labelled with a wide variety of luminescent probes are available from different sources. A preferred probe used in the carrier is a hydrophobic compound, having negligible solubility to water to avoid leakage. A variety of probes suitable for such labelling can be found amongst scintillator and laser dyes. With highly luminescent beads the great number of acceptor molecules may compensate the long distance after bead coating, and the luminescent bead actually provide a energy accepting surface. When the bead can absorb most of the donor emitted light, a simple radiative enery transfer can be applied, in which the energy transfer is a function of space angle and critical distance with 10 µm beads is in the range of micrometers. For FRET-based assay, however, when the plastic is first coated with the binding proteins (e.g. agglutinin) to immobilize membrane receptors, may result in inefficient energy transfer due to long distances. A preferred arrangement is thus to use surface activated beads and use part of the reactive groups for coupling with acceptor molecules, or use acceptor-labelled binding surface (such as rhodamine labelled agglutinin) or to label coated protein afterwards with acceptors.

In homogeneous assay of a association reaction (immunobinding, receptor-ligand binding, hybridizations reaction, enzyme-substrate binding etc.) the preferred way to measure binding is to follow acceptor signal increase. The acceptor signal is measured using a filter optimized for the donor used, having good transmission at the wavelength of acceptor, but more importantly, absolutely well blocked for each emission lines of the donor. The filter should not leak any emission emanating form the main emission line of donor (such as 545 or 490 nm of Tb and 613-615 nm of Eu). In addition the energy transfer filter has to be situated at wavelength area, where there are no minor emission lines with the used donor. Use of suitable delay avoid the interference derived from direct excitation of acceptor (the optimal delay depends on the length of

excitation pulse used, but should be at least ten times longer).

The decay of the energy transfer excited acceptor is a function of the decay of donor and the energy transfer efficiency. Thus, during the assay (such as competitive binding assay or non-competitive assay) the overall decay is not constant, but is a function of the analyte. In association assays where specific binding is low and energy transfer efficiency less than 1 %, the decay time of energy transfer emission of acceptor is quite constant and equal to the decay time of donor. The delay and counting times for such measurement is not critical. For assays of higher efficiencies, the decay time decreases upon binding, and steeper response can be obtained keeping short delay time and reasonable short counting time. On the other hand, if donor emission is followed, steeper response is obtained using long delay time, because when energy transfer occurs, the total donor emission both decreases and its decay time shortens. For optimized results in any assay, it is advisable to optimize counting windows according to assay type, specific binding percentages and energy transfer efficiencies.

In screening of various compounds or samples, the sample may have a signal decreasing effect. The sample may, for example, absorb excitation light, emission light or cause other types of quenchings. In a multi-label counter, such as Wallac 1420 VICTOR, the interferences can be controlled by various methods, including (1) decay analysis (2) simultaneous photometric monitoring of sample absorption at the wavelengths of excitation and emission, (3) simultaneous measurement of both signals, donor and acceptor, and calculating their correlation and comparing to control with same amounts of donor and acceptor without binding reaction or (4) kinetic measurement during binding following the change of energy transfer or signals. With binding assays of high efficiency, the analysis of decay

time is an independent parameter not affected by color quenching of neither the excitation nor emission. The decay change can be followed both for donor or for acceptor (decrease in decay when energy transfer increases). A preferred way to quarantee nondisturbed analysis with all samples is to use suitable combination of all the above mentioned correction methods.

According to a preferred embodiment, the energy transfer is registered by a time-resolved fluorometer using a predeterminated instrumental setting avoiding cross talk or donor emission to the channel used for acceptor emission measurement when there is no energy transfer.

Preferably, the instrument automatically corrects any attenuation of excitation the sample may cause by following simultaneously the absorbances of the samples diluted into assay mixture and correcting the emission readings according to excitation or emission attenuation by sample absorption.

The measured acceptor signal can be normalized by recording also the donor emission which can be compared to zero calibrator, the lowering of signal without concomitant increase in acceptor luminescence being recorded and corrected.

The decay time of either donor or the acceptor can be registered and used as the measuring parameter.

The decay time of the donor or the acceptor can be utilized in correction of sample interferences.

A second type of assay where improved energy transfer assays can be applied, are assays where dissociations, and not associations, are followed. Such assays can be e.g. competitive immunoassays (or other specific binding assay) where antibody-antigen complex is preformed and the

addition of analyte causes new equilibrium formations (ligand replacement assays). Another group of such assay are the assays of cleaving enzymes, such as peptidases (e.g. HIV peptidases), proteases and other enzymes (e.g. nucleotide hydrolyzing enzymes, helicase, etc.). In homogeneous measurement of dissociation, the preferred technology is to follow the increased emission of the donor.

Preferred chelates for assays based on dissociation are to a great extend same as described above for association based assays. Terbium, however, is often a better choice, because it can be quenched with a variety of compounds, including metal ions, nitrogen compounds such as azide, nitride (Tanaka et al, J Photochem Photobiol 74; 15, 1993) or radicals (spin labels, such as Doxyl, Proxyl or Tempo and other N-O compounds containing unpaired electron) (Matko et al, Biochemistry 31; 703, 1992). Good Tb chelates suitable for the present invention are mentioned above.

A preferred acceptor for dissociation measuring assay is a good energy acceptor causing as efficient donor quenching as possible. The acceptor can be luminescent, but does not have to be. A preferred FRET acceptor has good acceptor properties (R_0 over 5 nm), but does not have to be highly luminescent. In addition to luminescent compounds mentioned above, the good acceptor can be a respective compound made non-luminescent with heavy atom conjugations (such as erythrosin) or suitable other substituents (see. e.g. Khanna and Ullman, Anal Biochem 108; 156, 1980, Ullman and Khanna, Methods Enzymol 74; 28, 1981). To avoid steric interference (e.g. with HIV peptides labelled both with donor and with acceptor), the acceptor has to be small, and small molecular quenchers, such as spin labels, are preferred. Use of larger acceptors possibly requires indirect approach, i.e. use of small affinity label (small hapten, biotin) and separate step for quenching (addition of acceptor labelled anti-hapten of avidin), which may have

to be done after the actual dissociation reaction (end point detection). A preferred assay design for e.g. a peptidase is to use peptide substrate labelled at one end with a fluorescent terbium chelate and at the other end with free radical. The choice between spin quenching and FRET dipole-dipole energy transfer depends on the actual distance between the labels in unhydrolyzed peptide. With small distances (below 2 nm) free radical (spin) is preferred. If the distance of the two probes would be more than 5 nm, a dipole-dipole energy transfer is preferred.

When designed to be quenched in associated form (such as quenched substrate), the donor luminescence, and its decay time is increased upon dissociation. So the preferred delay time in measurement is long, to avoid detecting of possible remaining luminescence of quenched donor. These assays, similar to association assays, are sometimes prone to sample interferences (e.g. color quenchings), and the problems can be solved similarly as described above.

EXAMPLES

The invention is further demonstrated by the following non-limiting examples.

Example 1

Labelling of anti-ßhCG (human chorionic gonadotropin) antibody (code F19-9C1) with the fluorescent Tb chelate Compound 6

The energy donor Compound 6 was conjugated to anti-ßhCG antibody (code F19-9C1, Wallac Oy, Turku, Finland) in the following way: anti-ßhCG antibody (5mg/ml) was incubated with a 30-fold molar excess of Compound 6 in 50mM carbonate buffer pH 9.5 over night at +4 °C. The labelled antibodies were separated from unreacted Compound 6 by gel filtration (Sepharose 6B with Sephadex G50 overlay, 0.5x70 cm,

Pharmacia, Uppsala, Sweden) with Tris (50 mM Tris-HCl, pH 8 containing 0.9% NaCl) as eluetion buffer. The labelled antibody was analyzed and found to contain 4.7 molecules of Compound 6 per antibody. Labelled antibody was kept in Tris buffer, pH 7.4 containing 0.1% bovine serum albumin.

Example 2

Labelling of anti-ßhCG (code M15294) antibody with tetramethylrhodamine isothiocyanate (TRITC)

The energy acceptor TRITC (Molecular Probes Inc, Eugene, OR) was conjugated to anti-ßhCG antibody (code M15294, Wallac Oy) in the following way: anti-ßhCG antibody (5 mg/ml) was incubated with a 15-fold molar excess of TRITC in 50 mM carbonate buffer pH 9.5 over night at +4 °C. The labelled antibodies were separated from unreacted TRITC by gel filtration (Sepharose 6B with Sephadex G50 overlay, 0.5x70 cm, Pharmacia) with Tris (50 mM Tris-HCl, pH 8 containing 0.9% NaCl) as eluetion buffer. The labelled antibody was analyzed and found to contain 1,7 tetramethylrhodamine per antibody. Labelled antibody was kept in Tris buffer, pH 7.4 containing 0.1% bovine serum albumin.

Example 3

Homogenous energy transfer assay of ßhCG

In this two-site immunoassay, 50 µl of ßhCG standard in the wells of 96-well plates was incubated with 100 ng of Compound 6 -labelled antibody (donor) and 100 ng of tetramethylrhodamine labelled antibody (acceptor) in 200 µl Tris buffer (50 mM Tris-HCl, pH 7.4, containing 0.9 % NaCl). The two anti-ßhCG antibodies used are directed against different specific antigenic sites on the ßhCG subunit. The ßhCG standards were from a commercially available kit for the measurement of free ßhCG in serum

(Wallac Oy). The reaction mixtures were incubated for 30 min at room temperature on a 96-well plate shaker. The tetramethylrhodamine fluorescence from the formed complexes was measured in a time-resolved fluorometer (excitation at 340 nm, emission at 572 nm +- 3 nm, delay time 50 µs, window time 100 µs, cycle time 1 ms), model 1420 VICTOR (Wallac Oy) A standard curve for the energy transfer assay of ßhCG is presented in Figure 3.

Example 4

Homogenous energy transfer assay of PSA (prostate specific antigen)

Two antibodies (Wallac Oy) recognising different epitopes on PSA were labelled with Compound 1 and TRITC, respectively. Antibody labelling were carried out as described in example 1 and 2. In this two-site immunoassay, 50 µl of PSA standard (from the commercially available PSA assay kit DELFIA PSA) was incubated together with 100 ng of Compound 1 (donor) labelled antibody and 100 ng of tetramethylrhodamine labelled antibody (acceptor) in 200 µl Tris buffer pH 7.4. The assay mixtures were incubated for 30 min at room temperature under continous shaking. The tetramethylrhodamine fluorescence from the formed complexes (sandwiches) was measured in the Wallac 1420 VICTOR time-resolved fluorometer (Excitation at 340 nm, emission at 572 nm +- 3 nm, delay time 50 µs, window time 100 µs, cycle time 1 ms). The standard curve for the homogenous energy transfer assay is documented in Figure 4.

Example 5

Homogenous energy transfer assay of ßhCG with tetramethyl-rhodamine-avidin as acceptor

This two-site immunoassay was performed as described in

Example 3. Instead of using tetramethylrhodamine-labelled antibody (acceptor), biotinylated antibody and tetramethylrhodamine-avidin (acceptor) was used. The tetramethylrhodamine-avidin (Molecular Probes) reacts and forms stable complexes with the biotinylated antibody. The anti-ßhCG antibody (code M15294) was biotinylated according to the instructions from the biotin reagent supplier (Biotinylating Reagent 732494, Boehringer Mannheim, Mannheim, Germany). The tetramethylrhodamine fluorescence from the formed complexes (sandwiches) was measured in the Wallac 1420 VICTOR time-resolved fluorometer (excitation at 340 nm, emission at 572 nm +- 3 nm, delay time 50 µs, window time 100 µs, cycle time 1 ms). The standard curve for the homogenous energy transfer assay is documented in Figure 5.

Example 6

Homogenous energy transfer assay of ßhCG with R-phycoerythrin-avidin as acceptor

In this experiment R-phycoerythrin-avidin (Molecular Probes, Eugene, OR, USA) was used instead of tetramethylrhodamine-avidin, otherwise the experiment was performed as in Example 5. The R-phycoerythrin fluorescence from the formed complexes (sandwiches) was measured in the Wallac 1240 VICTOR time-resolved fluorometer (Excitation at 340 nm, emission at 572 nm +-3 nm, delay time 50 µs, window time 100 µs, cycle time 1 ms). The standard curve for the homogenous energy transfer assay is documented in Figure 6.

Example 7

Homogenous energy transfer assay of progesterone

Monoclonal anti-progesterone antibody (Wallac Oy) was labelled with Compound 2 according to Example 1. Progesterone-biotin derivative (Wallac Oy) was used at a

concentration of $5/K_{aff}$ (K_{aff} = affinity constant of the progesterone antibody used) and about 2 times more tetramethylrodamin-avidin (from Molecular Probes, Inc, OR) was used. Tris buffer was used in the incubations. In this competitive immunonassay 50 µl standard or sample, was incubated 100 µl of Compound 2 -labelled antibody (diluted 1/50000) and 100 µl of progesterone-biotin derivative and tetramethylrodamin-avidin (acceptor). The biotinylated progesterone competes with progesterone from the standards or the sample for the binding sites on the labelled anti-progesterone antibody. The reaction mixture was incubated for 1 hour at room temperature. After the incubation the fluorescence signal was measured from tetramethylrodamin-avidin bound to the anti-progesterone-antibody-progesterone-biotin complexes with a time-resolved fluorometer (Wallac 1420 VICTOR, excitation at 340 nm, emission at 572 nm, +- 3 nm, delay time 50 µs, window time 100 µs, cycle time 1 ms). The measured signal is inversely related to the progesterone concentration in the sample.

Example 8

Homogenous energy transfer receptor assay of IL-2

Purified carrier-free IL-2 (R&D Systems, Minneapolis, MN) (1 mg/ml) was incubated with a 10-fold molar excess of Compound 2 in 50mM carbonate buffer pH 9.2 over night at + 4 °C. The labelled IL-2 was separated from unreacted chelate by gel filtration (Sephadex G50, 0.7x48 cm, Pharmacia) with Tris as elution buffer. The labelled IL-2 was analyzed and found to contain 1.4 molecules of Compound 2 per IL-2 molecule. A non-neutralizing antibody directed against the IL-2α receptor was labelled with tetramethylrhodamine isothiocyanate according to Example 2. In the homogenous receptor assay increasing concentrations of unlabelled IL-2 was co-incubated with constant quantities of Compound 2 -labelled IL-2 (donor), IL-2α

receptor and the tetramethylrhodamine labelled antibody (acceptor). Tris buffer was used in this incubation. A displacement curve was constructed by measuring the fluorescence signal from the formed antibody-receptor-IL-2 complexes in a time-resolved fluorometer (Wallac 1420 VICTOR, excitation at 340 nm, emission at 572 nm +- 3 nm, delay time 50 µs, window time 100 µs, cycle time 1 ms).

Example 9

Double labelling of casein with a Compound 6 and teramethylrhodamine

Five milligrams of casein was dissolved in 0.5 mL of 0.1 M sodium carbonate pH 9.2. Fifty microliters of 5.5 mM fluorescent Compound 6 in water was added to the casein solution and reaction was allowed to proceed overnight at 4 °C. The labelled protein was purified through Sephadex G-50 column using 0.9 % sodium chloride in aqueous solution as an eluent. The labelled casein was analyzed and found to contain one Compound 6 molecule per casein molecule. Two milligrams of Compound 6 -labelled casein in 1 mL was mixed with 100 µl of 20 mM tetramethylrhodamine isothiocyanate (TRITC) dissolved in dimethylformamide. After adjusting pH by adding 120 µl of 1 M sodium carbonate, pH 9.5, reaction mixture was incubated at 4 °C overnight. The labelled protein was purified from unreacted TRITC using Sephadex G-50 column equilibrated and run with 50 mM Tris-HCl pH 8 containing 0.9% NaCl. Using the same protocol as for Compound 6 -labelled casein, unlabelled casein was also labelled with TRITC.

Example 10

Labelling of Compound 6 -labelled casein with a spin label.

Two milligrams of Compound 6 -labelled casein (Example 9) in 1 mL 0.9% NaCl was mixed with 110 µl of 1 M sodium

carbonate pH 9.5 and 13 µl of 160 mM 3-(2-(2-Isothiocyanatoethoxy)-ethylcarbamoyl)-PROXYL (Aldrich) was added. After overnight reaction at 4 °C the double labelled protein was purified using Sephadex G-50 column.

Example 11

Fluorometric properties of double-labelled caseins

The double-labelled proteins from Examples 9 and 10 together with Compound 6 casein (Example 9) were analyzed in a multilabel reader (VICTOR, Wallac Oy). Labelled caseins were diluted in 50 mM Tris-HCl pH 7.8 to a concentration of 0.2 µg/mL. Using the protocol for terbium measurement (excitation 340 nm, emission 545 nm, delay time 0.5 ms, window time 1.4 ms, cycle time 2 ms) Compound 6-labelled casein gave 743 000 counts per second (cps). Casein labelled with Compound 6 and TRITC gave 28 000 cps, casein labelled with Compound 6 and PROXYL 54 000 cps and casein labelled with TRITC 869 cps. When the abovementioned proteins were measured using excitation at 340 nm, emission at 572 nm +- 3nm (suitable for TRITC), delay time 50 µs, window time 100 µs and cycle time 1 ms, Compound 6 -labelled casein gave 3952 cps, Compound 6- and TRITC-labelled casein 382 000 cps, Compound 6- and PROXYL-labelled casein 4097 cps and TRITC-labelled casein 747 cps. These results show that labelling of Compound 6-labelled casein with TRITC or PROXYL efficiently quenched Compound 6 -fluorescence. In the case of Compound 6- and TRITC-labelled casein quenching of Compound 6 -fluorescence is due to energy transfer between Compound 6 -chelate and rhodamine which can be seen in fluorescence signal using an emission filter suitable for rhodamine and time-resolved mode.

Example 12

Protease assay using double-labelled caseins

Compound 6 -labelled casein, Compound 6- and TRITC-labelled casein and Compound 6- and PROXYL-labelled casein were diluted in 10 mM Tris-HCl pH 7.6 to a concentration of 50 ng/mL. 180 µl of each diluted protein was pipetted in clear microtitration wells (NUNC Maxisorp). Fluorescence was measured using the protocol for terbium measurement. Following results were obtained: Compound 6 -casein 179 000 cps, Compound 6 -TRITC-casein 7860 cps and Compound 6 -PROXYL-casein 14680 cps. Then 100 ng of trypsin in 20 µl of 10 mM Tris-HCl pH 7.6 was added to each well. After incubating for 1 hour at room temperature Compound 6 -fluorescence was measured: Compound 6 -casein 163 000 cps, Compound 6 -TRITC-casein 137 000 cps and Compound 6 -PROXYL -casein 67400 cps. In case of double-labelled caseins there was up to 17-fold increase in Compound 6 -fluorescence upon degradation with a protease.

Example 13

Homogeneous energy transfer assay for measurement of a protein kinase activity

Antibody against phosphorylated tyrosine (clone PY20) was labelled with Compound 4. Incorporation of 3 chelates per antibody molecule was obtained. Rhodamine-avidin (Rh-Av) and phycoerythrin-avidin (PE-Av) were purchased from Vector Laboratories. As a substrate we used a peptide with a following amino acid sequence: Biotin-Glu-Ala-Ile-Tyr-Ala-Ala-Pro-Phe-Ala-Lys-Lys-Lys. The biotin moiety was coupled to the amino terminus of the peptide. This peptide is a substrate for Abl protein tyrosine kinase purchased from New England Biolabs. The assay was started by incubating 100 nM peptide solution with labelled avidin (concentration 10 µg/mL). Twenty

microliters of peptide-avidin solution was pipetted to clear microtitration wells. Next 75 µL of Abl protein tyrosine kinase assay buffer (New England Biolabs; 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 0.1 mM Na_2EDTA, 1 mM dithiothreitol, 0.015% Brij 35) containing 25 µM ATP and 250 µg/mL Compound 3 -labelled anti-phosphotyrosine was added to each well. Then either 5 µL of kinase assay buffer (blank) or 5 µL of Abl protein tyrosine kinase (1 unit) diluted in assay buffer was added. Measurement was performed in VICTOR reader using time-resolved mode (excitation at 340 nm, emission at 572 nm +- 3 nm, delay time 50 µs, window time 100 µs, cycle time 1 ms) to measure signal from rhodamine or phycoerythrin excited by energy transfer from Compound 4. After 30 minutes following results were obtained:

Labelled avidin	Blank	Signal with enzyme
Rhodamine-avidin	2649 cps	702 000 cps
Phycoerythrin-avidin	2460 cps	974 000 cps

These results show that kinase activity can be detected using the described assay format based on energy transfer between Compound 4 and rhodamine or phycoerythrin.

Example 14

Homogeneous energy transfer DNA hybridization assay

The following oligonucleotides were synthesized using Applied Biosystems DNA/RNA synthesizer:
Target oligonucleotide 5'-ACG GTT CTA GCG TTG CAC TAC GGC TGC AAC TAG CAG TCC-3'
Compound 6 -labelled oligonucleotide 5'-Compound 3-X-GTG CAA CGC TAG AAC-3'
Tetramethylrhodamine-labelled oligonucleotide 5'-TGC TAG TTG CAG CCG-X-TRITC-3'
Compound 6- and TRITC-labelled oligonucleotides were

synthesized and labelled using the chemistry and protocol published earlier (P. Dahlen et al. (1994) Bioconjugate Chem. 5, 268-272). X denotes a modified deoxycytidine residue carrying an aliphatic primary amino group suitable for labelling with reagents containing, for example, isothiocyanate group. Compound 6 used in this example is fluorescent. The labelled probes are able to hybridize with the target oligonucleotide so that upon hybridization the fluorescent Compound 6 and tetramethylrhodamine are separated only by two nucleotides in the target oligonucleotide. In the assays following hybridization solution was used: 0.6 M sodium chloride, 50 mM Tris-HCl pH 8, 0.1 mM EDTA, 0.1% sodium dodecylsulphate. The probes were diluted in hybridization solution to give the final concentration of 10 nM each. The target oligonucleotide was diluted also in hybridization buffer to give the following concentrations: 1 nM (in the assay 0.1 nM), 5 nM (in the assay 0.5 nM) and 10 nM (in the assay 1 nM). Hybridization solution alone was used as a target for blank. Twenty microliters of target oligonucleotide dilutions and blank was pipetted to clear microtitration wells. The assay was performed using two replicates. Then 180 µl of hybridization solution containing the labelled probes was added to each well. Then the plate was covered with adhesive tape and placed in an incubator set to 37 °C. After incubation for 30 minutes the plate was measured using the time-resolved mode (excitation at 340 nm, emission at 572 nm +- 3 nm, delay time 50 µs, window time 100 µs, cycle time 1 ms). Following results were obtained:

Blank	2331 cps
0.1 nM target	7170 cps
0.5 nM target	26980 cps
1 nM target	46300 cps

These results show that this homogeneous assay works well and the energy transfer between the Compound 6 and rhodamine is efficient when the two probes hybridize to the

same target molecule.

Example 15

Energy transfer assay using beads containing acceptor molecules

Anti-ßhCG antibody (code M15294) was labelled with Compound 6 as described in Example 1. Fifty microliter of orange fluorescent beads ($1.8x10^6$ beads/ml) (FluoroSpheres®, F-8857 from Molecular Probes Inc.) were incubated with 50 µl Compound 6 -labelled antibody (60 µg/ml) in Tris buffer (50 mM Tris-HCl, pH 7.4 containing 0.9% NaCl) for 2 hours. In the reaction mixture Compound 6 -labelled antibody is passively absorbed to the surface of the bead. The background was determined by incubating 50 µl of beads with 50 µl buffer and 50 µl of antibody solution with 50 µl of buffer. The fluorescence from the acceptor molecules in the beads excited by energy transfered from Compound 6 was measured in the Wallac 1240 VICTOR time-resolved fluorometer. Fluorometer setting used were: excitation at 340 nm, emission at 572 nm +- 3nm, delay time 50 µs, window time 100 µs, cycle time 1 ms. Results:

	Fluorescence 1000xcps
50 µl beads + 50 µl Compound 6 -labelled antibody	5543
50 µl beads + 50 µl buffer	161
50 µl Compound 6 -labelled antibody + 50 µl buffer	158

Example 16

Energy transfer assay using beads containing acceptor molecules

Anti-ßhCG antibody (code M15294) was labelled with Compound 9 as described in Example 1. Fifty microliter of crimson fluorescent beads ($1.8x10^6$ beads/ml) (FluoroSpheres®),

F-8855 from Molecular Probes Inc.) were incubated with 50 µl Compound 9 -labelled antibody (60 µg/ml) in Tris buffer (50 mM Tris-HCl, pH 7.4 containing 0.9% NaCl) for 2 hours. In the reaction mixture Compound 9 -labelled antibody is passively absorbed to the surface of the bead. The background was determined by incubating 50 µl of beads with 50 µl buffer and 50 µl of antibody solution with 50 µl of buffer. The fluorescence from the acceptor molecules in the beads excited by energy transfered from Compound 9 was measured in the Wallac 1240 VICTOR time-resolved fluorometer. Fluorometer setting used were: excitation at 340 nm, emission at 660 nm +- 1 nm, delay time 100 µs, window time 100 µs, cycle time 1 ms. Results:

	Fluorescence 1000xcps
50 µl beads + 50 µl Compound 9 -labelled antibody	1398
50 µl beads + 50 µl buffer	8
50 µl Compound 9 -labelled antibody + 50 µl buffer	102

It will be appreciated that the methods of the present invention can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. It will be apparent to the artisan that other embodiments exist and do not depart from the spirit of the invention. Thus, the described embodiments are illustrative and should not be construed as restrictive.

CLAIMS

1. A luminescence energy transfer assay comprising a first group labelled with an energy donor and a second group labelled with an energy acceptor, wherein
- the donor is a long excited state lifetime luminescent lanthanide chelate and the acceptor is either a short excited state lifetime luminescent label or a non-luminescent label, and
- and the increase or decrease, respectively, in energy transfer from the donor label to the acceptor label resulting from shortening or lengthening, respectively, of the distance between said labels, is measured
characterized in that the long excited state lifetime luminescent lanthanide chelate has one or more of the following properties:
- a high luminescence yield,
- an excited state lifetime of 1 ms or more, and
- an emission distribution optimized for energy transfer.

2. A luminescence energy transfer assay comprising a first group labelled with an energy donor and a second group labelled with an energy acceptor, wherein
- the acceptor is either a short excited state lifetime luminescent label or a non-luminescent label, and
- and the decrease in energy transfer from the donor label to the acceptor label resulting from lengthening of the distance between said labels, is measured
characterized in that the donor is a long excited state lifetime luminescent lanthanide chelate.

3. A luminescence energy transfer assay comprising a first group labelled with an energy donor and a second group labelled with an energy acceptor, wherein
- the acceptor is either a short excited state lifetime luminescent label or a non-luminescent label, and
- and the increase or decrease, respectively, in energy

transfer from the donor label to the acceptor label resulting from shortening or lengthening, respectively, of the distance between said labels, is measured characterized in that the donor is a luminescent lanthanide chelate, and

- two or more analytes are simultaneously measured, wherein each analyte is measured by a specific donor-acceptor pair and the donor and acceptor are selected from those defined above.

4. A luminescence energy transfer assay comprising a first group labelled with an energy donor and a second group labelled with an energy acceptor, wherein

- the acceptor is either a short excited state lifetime luminescent label or a non-luminescent label, and
- and the increase or decrease, respectively, in energy transfer from the donor label to the acceptor label resulting from shortening or lengthening, respectively, of the distance between said labels, is measured characterized in that the donor is a luminescent lanthanide chelate, and
- the luminescent or non-luminescent label of the acceptor is attached to a solid carrier providing a large surface to which said second group is bound or can be bound via suitable covalent or non-covalent means.

5. The assay according to claim 4 characterized in that the luminescent or non-luminescent label molecules are in the solid carrier.

6. The assay according to claim 4 characterized in that the solid carrier is surface activated microbeads covalently coupled with the second group and with a plurality of acceptor molecules.

7. The assay according to claim 4 characterized in that the surface has been made by coating the microbeads with an adherent macromole such as a polylysine, agglutinin,

lectin, antibody, which macromolecule either prior to or after the coating step is conjugated with the acceptor molecules.

8. A luminescence energy transfer assay comprising a first group labelled with an energy donor and a second group labelled with an energy acceptor, wherein
- the acceptor is either a short excited state lifetime luminescent label or a non-luminescent label, and
- and the increase or decrease, respectively, in energy transfer from the donor label to the acceptor label resulting from shortening or lengthening, respectively, of the distance between said labels, is measured
characterized in that the donor is a luminescent lanthanide chelate, and
- the acceptor is lipophilic compound anchored to a cell membrane or cell membrane fragment or the acceptor is made lipophilic by conjugating with a long chain hydrocarbon such as a fatty acid.

9. The assay according to any of the claims 2 - 8 characterized in that the luminescent lanthanide chelate has a long excited state lifetime and that it has one or more of the following properties:
- a high luminescence yield,
- an excited state lifetime of 1 ms or more, and
- an emission distribution optimized for energy transfer.

10. The assay according to any of the claims 1 and 3 - 9 characterized in that the assay is based on a bioaffinity binding reaction.

11. The assay according to any of the claims 1 - 10 characterized in that the assay is based on an enzymatically catalyzed reaction.

12. The assay according to claim 11 characterized in that the reaction is a cleavage reaction.

13. The assay according to any of the foregoing claims characterized in that the lanthanide of the donor chelate is europium, terbium or samarium.

14. The assay according to claim 13 characterized in that the energy transfer is registered by a time-resolved fluorometer using a predeterminated instrumental setting avoiding cross talk or donor emission to the channel used for acceptor emission measurement when there is no energy transfer.

15. The assay according to claim 14 characterized in that the instrumental counting time window is optimized for the assay, having delay period long compared to the length of excitation pulses and the emission lifetime of the acceptor without energy transfer, and the counting window shorter than the decay of the donor in assays measuring decreased distances.

16. The assay according to any of the foregoing claims characterized in that the acceptor molecules are brought to the vicinity of specific binding reaction using suitable secondary affinity reactions, such as anti-receptor antibodies, use of biotinylated binding reagents and acceptor labeled avidin, lectins etc.

17. The assay according to any of the claims 1 - 9 or 11 - 16 characterized in that the acceptor is a spin label.

18. A luminescence energy transfer assay comprising a first group labelled with an energy donor and a second group labelled with an energy acceptor, wherein
- the acceptor is either a short excited state lifetime luminescent label or a non-luminescent label, and
- and the increase or decrease, respectively, in energy transfer from the donor label to the acceptor label resulting from shortening or lengthening, respectively, of the distance between said labels, is measured

characterized in that the donor is a luminescent lanthanide chelate, and that the instrument used to measure energy transfer is able to follow multiple parameters simultaneously or consecutely, such as absorbances at the wavelenghts of excitation or emissions, luminescence of different durations, scattering and luminescence decay times, in order to monitor and correct any interferences the sample matrix may have to excitation, energy transfer or emissions.

19. The assay according to claim 18 characterized in that the instrument automatically corrects any attenuation of excitation the sample may cause by following simultaneously the absorbances of the samples diluted into assay mixture and correcting the emission readings according to excitation or emission attenuation by sample absorption.

20. The assay according to claim 18 characterized in that the measured acceptor signal is normalized by recording also the donor emission which can be compared to zero calibrator, the lowering of signal without concomitant increase in acceptor luminescence being recorded and corrected.

21. The assay according to claim 18 characterized in that the decay time of either donor or the acceptor is registered and used as the measuring parameter.

22. The assay according to claim 18 characterized in that the decay time of the donor or the acceptor is utilized in correction of sample interferences.

23. The assay according to claim 18 characterized in that it is a suitable combination of the measurements according to claims 19, 20, 21 or 22.

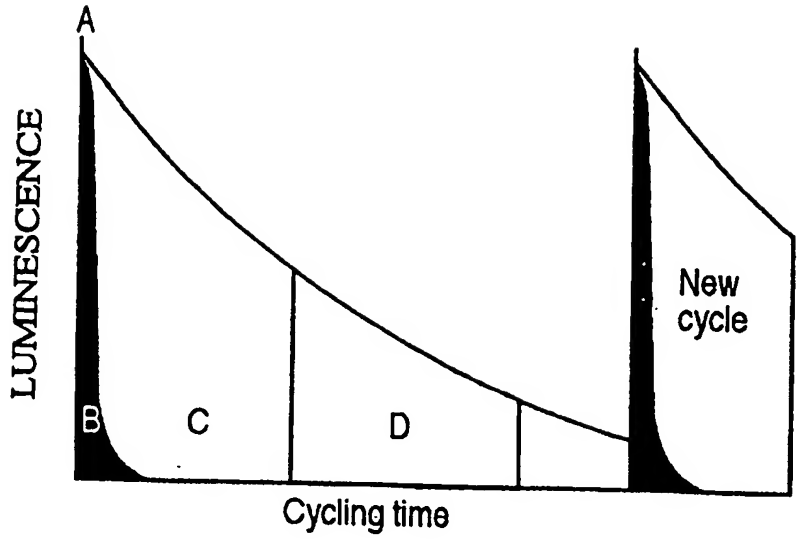

A
LUMINESCENCE
B
C
D
New
cycle
Cycling time

FIG. 1

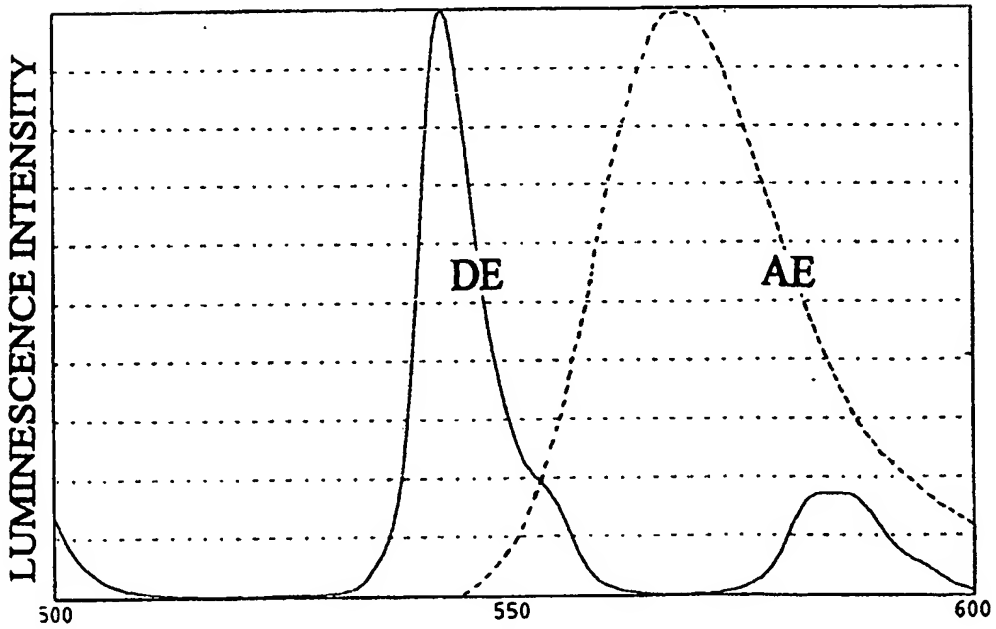

LUMINESCENCE INTENSITY
DE
AE
500
550
600
EMISSION WAVELENGTH

FIG. 2

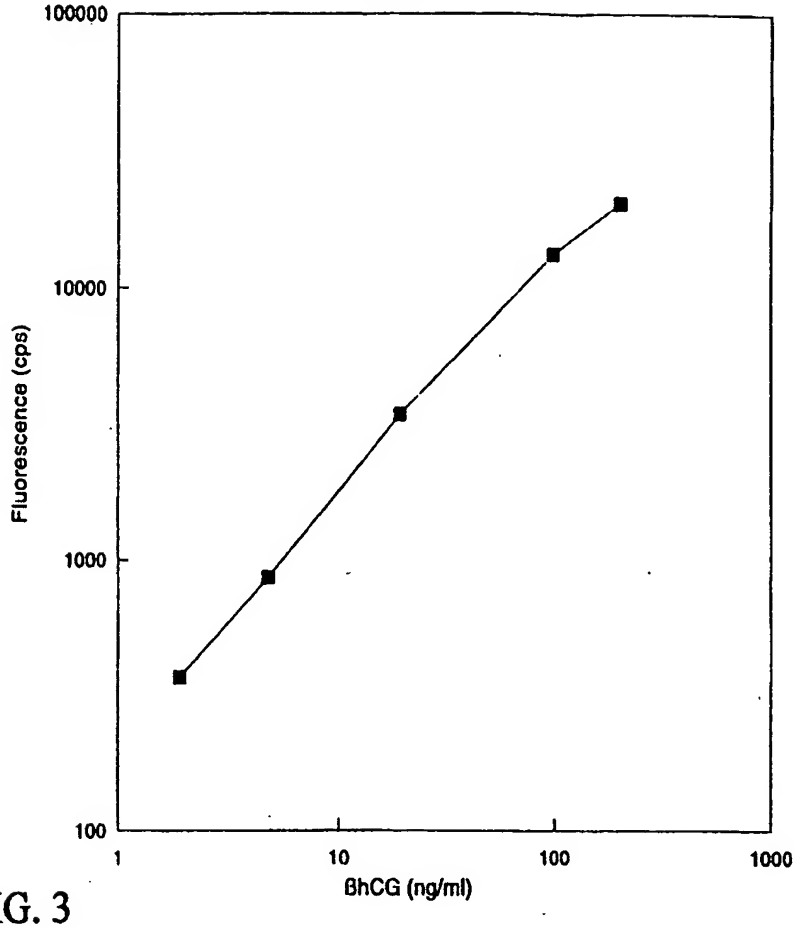

100000
10000
1000
100
Fluorescence (cps)
1
10
100
1000
βhCG (ng/ml)

FIG. 3

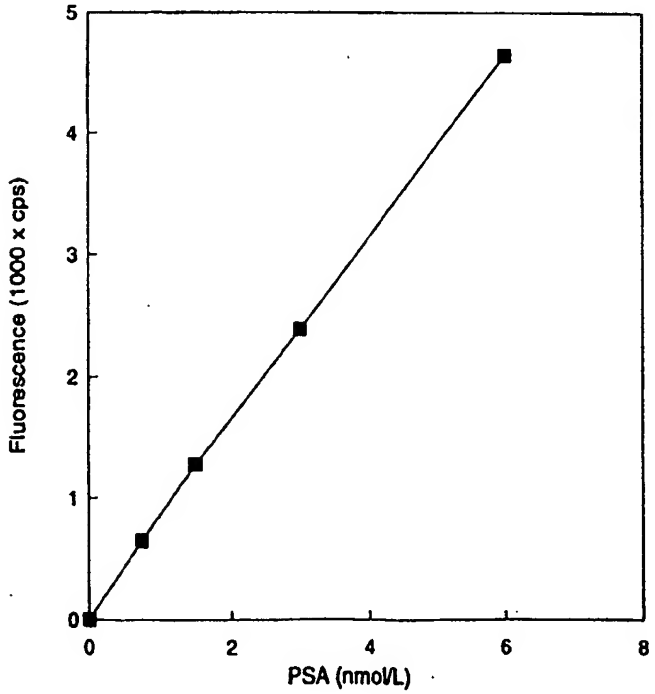

5
4
3
2
1
0
Fluorescence (1000 x cps)
0
2
4
6
8
PSA (nmol/L)

FIG. 4

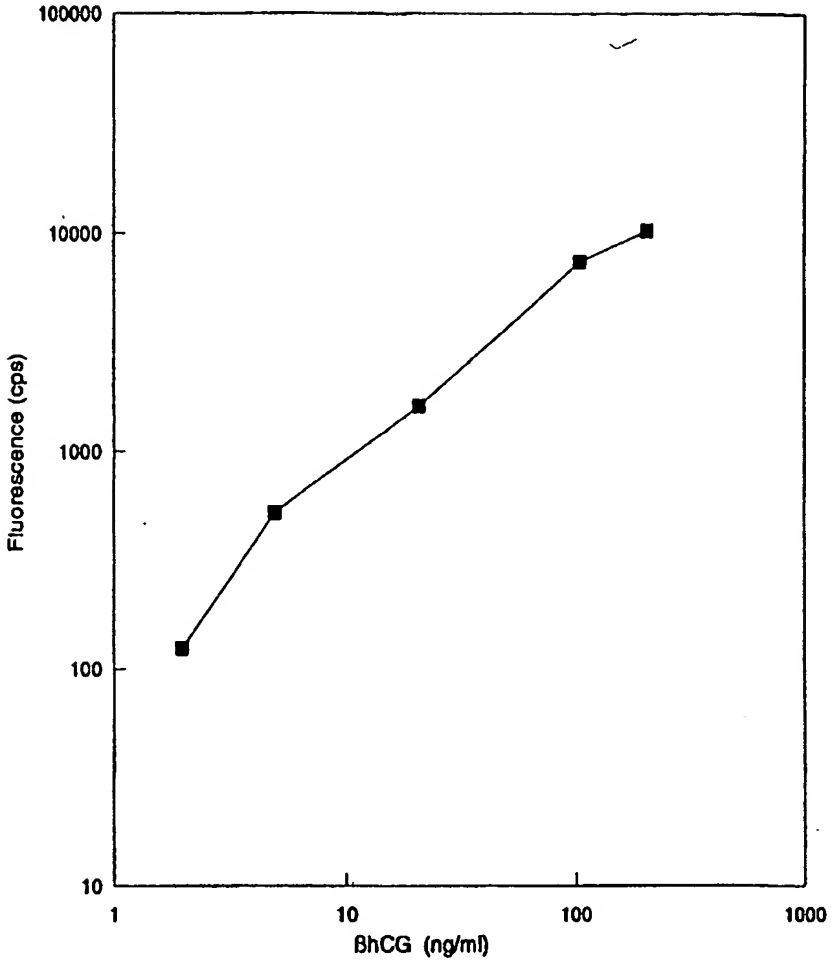

100000
10000
1000
100
10
Fluorescence (cps)
1
10
100
1000
βhCG (ng/ml)

FIG. 5

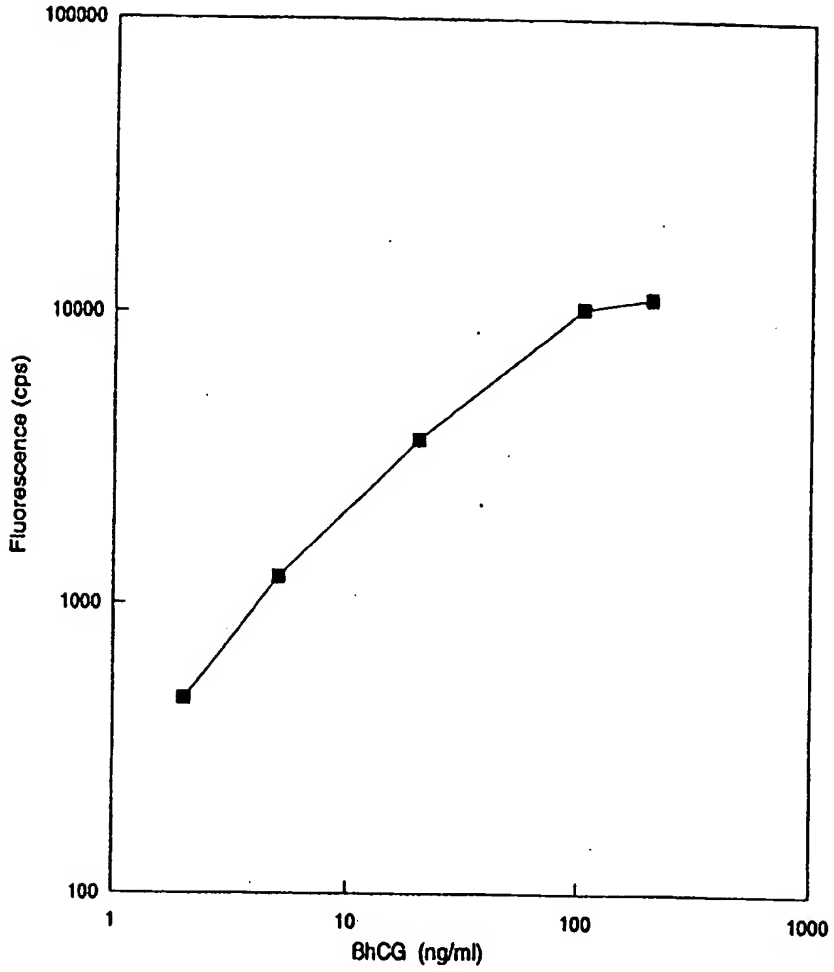

100000
10000
1000
100
Fluorescence (cps)
1
10
100
1000
βhCG (ng/ml)

FIG. 6